Patents Form 1/77

The Patent Office

D Young & Co

Patents Act 1977
(Rule 16)

THE PATENT OFFICE
31 MAR 1998
RECEIVED BY HAND

19FEB98 E349262-1 [illegible]
P01/7700 25.00 - 9806895.

Request for a grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

1. Your reference — P4287.GB ATM

2. Patent application number
(The Patent Office will fill in this part) — 31 MAR 1998 — **9806895.0**

3. Full name, address and postcode of the or of each applicant
(underline all surnames) — MEDICAL RESEARCH COUNCIL
20 PARK CRESCENT,
LONDON,
W1N 4AL.

Patents ADP number *(if you know it)* — 5916007001

If the applicant is a corporate body, give the country/state of its incorporation — BRITISH

4. Title of the invention — NUCLEIC ACID BINDING PROTEINS

5. Name of your agent *(if you have one)* — D YOUNG & CO

"Address for service" in the United Kingdom to which all correspondence should be sent
(including the postcode) — 21 NEW FETTER LANE
LONDON
EC4A 1DA

Patents ADP number *(if you have one)* — 59006

6. If you are declaring priority from one or more earlier patent applications, give the country and date of filing of the or each of these earlier applications and (if you know it) the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day/month/year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and filing date of the earlier application

Number of earlier application	Date of filing *(day/month/year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*
a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

YES

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	
Description	29
Claims(s)	5
Abstract	1
Drawing(s)	4

184

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature

Date 31.03.1998

D YOUNG & CO
Agents for the Applicants

12. Name and daytime telephone number of the person to contact in the United Kingdom

DR. A. MASCHIO 01703 634816

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505

b) Write your answers in capital letters using black ink or you may type them

c) If there is not enough space for all the relevant details on any part of this form, please continue on a separte sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.

d) If you answered 'Yes' Patents Form 7/77 will need to be filed.

e) Once you have filled in the form you must remember to sign and date it.

f) For details of the fee and ways to pay please contact the Patent Office.

Nucleic Acid Binding Proteins

The present invention relates to DNA binding proteins. In particular, the invention relates to a method for designing a protein which is capable of binding to a defined methylated DNA sequence but not to an equivalent unmethylated DNA sequence.

Protein-nucleic acid recognition is a commonplace phenomenon which is central to a large number of biomolecular control mechanisms which regulate the functioning of eukaryotic and prokaryotic cells. For instance, protein-DNA interactions form the basis of the regulation of gene expression and are thus one of the subjects most widely studied by molecular biologists.

A wealth of biochemical and structural information explains the details of protein-DNA recognition in numerous instances, to the extent that general principles of recognition have emerged. Many DNA-binding proteins contain independently folded domains for the recognition of DNA, and these domains in turn belong to a large number of structural families, such as the leucine zipper, the "helix-turn-helix" and zinc finger families.

Despite the great variety of structural domains, the specificity of the interactions observed to date between protein and DNA most often derives from the complementarity of the surfaces of a protein α-helix and the major groove of DNA [Klug, (1993) Gene 135:83-92]. In light of the recurring physical interaction of α-helix and major groove, the tantalising possibility arises that the contacts between particular amino acids and DNA bases could be described by a simple set of rules; in effect a stereochemical recognition code which relates protein primary structure to binding-site sequence preference.

It is clear, however, that no code will be found which can describe DNA recognition by all DNA-binding proteins. The structures of numerous complexes show significant differences in the way that the recognition α-helices of DNA-binding proteins from

different structural families interact with the major groove of DNA, thus precluding similarities in patterns of recognition. The majority of known DNA-binding motifs are not particularly versatile, and any codes which might emerge would likely describe binding to a very few related DNA sequences.

Even within each family of DNA-binding proteins, moreover, it has hitherto appeared that the deciphering of a code would be elusive. Due to the complexity of the protein-DNA interaction, there does not appear to be a simple "alphabetic" equivalence between the primary structures of protein and nucleic acid which specifies a direct amino acid to base relationship.

International patent application WO 96/06166 addresses this issue and provides a "syllabic" code which explains protein-DNA interactions for zinc finger nucleic acid binding proteins. A syllabic code is a code which relies on more than one feature of the binding protein to specify binding to a particular base, the features being combinable in the forms of "syllables", or complex instructions, to define each specific contact.

Our copending UK patent applications, GB 9710805.4, 9710806.2, 9710807.0, 9710808.8, 9710809.6, 9710810.4, 9710811.2 and 9710812.0 describe improved techniques for designing zinc finger polypeptides capable of binding desired nucleic acid sequences. In combination with selection procedures, such as phage display, set forth for example in WO 96/06166, these techniques enable the production of zinc finger polypeptides capable of recognising practically any desired sequence.

Zinc finger domains studied and produced to date are capable of binding to recognition sequences composed by any of four nucleic acid bases: A, C, G or T (U in RNA). However, the DNA of many organisms includes also a fifth base, 5-methylcytosine (5-MeC or, in nucleotide sequences herein, M). 5-MeC arises from specific methylation of cytosine, and is used to mark the genome or to increase its information content. 5-methylcytosine is well known to affect protein-DNA interactions, for

instance inhibiting cleavage of DNA by certain restriction enzymes. In vertebrates, cytosine is frequently methylated when directly preceding guanine, as in the dinucleotide CpG. This type of methylation generally down-regulates vertebrate gene expression, and can also prevent the binding of many eukaryotic transcription factors to DNA. Yet the zinc finger transcription factors tested to date, Sp1 and YY1, are not affected by CpG methylation of their DNA binding sites, suggesting that zinc fingers are incapable of discriminating between cytosine and 5-meC.

Since methylated cytosine bases are involved with many regulatory interactions in gene expression, and particularly in eukaryotic, including human, gene expression, the production of zinc finger polypeptides which specifically target methylated cytosine bases would be highly desirable. Such polypeptides, in order to be useful, must be able to differentiate DNA sequences in which cytosine is methylated to 5-meC from identical non-methylated sequences.

Summary of the Invention

We have now determined that 5-meC can be specifically recognised, over unmethylated cytosine, by zinc finger polypeptides. The invention accordingly provides a method for producing a zinc finger polypeptide which binds to a target nucleic acid sequence containing a modified nucleic acid base, but not to an identical sequence containing the equivalent unmodified base.

In the present invention, a "modified" base is a nucleic acid base other than A, C, G or T as they occur in DNA in nature. Thus, the term modified includes methylated bases, such as 5-meC which occurs naturally in DNA, and base analogues, including naturally-occurring analogues such as U and artificial analogues such as I.

In a first embodiment, the invention provides a method for preparing a DNA binding polypeptide of the Cys2-His2 zinc finger class capable of binding to a DNA triplet in target DNA sequence comprising 5-meC as the central residue in the target DNA

triplet, wherein binding to the 5-meC residue by an α-helical zinc finger DNA binding motif of the polypeptide is achieved by placing an Ala residue at position +3 of the α-helix of the zinc finger.

All of the DNA-binding residue positions of zinc fingers, as referred to herein, are numbered from the first residue in the α-helix of the finger, ranging from +1 to +9. "-1" refers to the residue in the framework structure immediately preceding the α-helix in a Cys2-His2 zinc finger polypeptide. Residues referred to as "++" are residues present in an adjacent (C-terminal) finger. Where there is no C-terminal adjacent finger, "++" interactions do not operate.

Cys2-His2 zinc finger binding proteins, as is well known in the art, bind to target nucleic acid sequences via α-helical zinc metal atom co-ordinated binding motifs known as zinc fingers. Each zinc finger in a zinc finger nucleic acid binding protein is responsible for determining binding to a nucleic acid triplet in a nucleic acid binding sequence. Preferably, there are 2 or more zinc fingers, for example 2, 3, 4, 5 or 6 zinc fingers, in each binding protein. Advantageously, there are 3 zinc fingers in each zinc finger binding protein.

The method of the present invention allows the production of what are essentially artificial DNA binding proteins. In these proteins, artificial analogues of amino acids may be used, to impart the proteins with desired properties or for other reasons. Thus, the term "amino acid", particularly in the context where "any amino acid" is referred to, means any sort of natural or artificial amino acid or amino acid analogue that may be employed in protein construction according to methods known in the art. Moreover, any specific amino acid referred to herein may be replaced by a functional analogue thereof, particularly an artificial functional analogue. The nomenclature used herein therefore specifically comprises within its scope functional analogues of the defined amino acids.

The α-helix of a zinc finger binding protein aligns antiparallel to the nucleic acid strand, such that the primary nucleic acid sequence is arranged 3' to 5' in order to correspond with the N terminal to C-terminal sequence of the zinc finger. Since nucleic acid sequences are conventionally written 5' to 3', and amino acid sequences N-terminus to C-terminus, the result is that when a nucleic acid sequence and a zinc finger protein are aligned according to convention, the primary interaction of the zinc finger is with the - strand of the nucleic acid, since it is this strand which is aligned 3' to 5'. These conventions are followed in the nomenclature used herein. It should be noted, however, that in nature certain fingers, such as finger 4 of the protein GLI, bind to the + strand of nucleic acid: see Suzuki *et al.*, (1994) NAR 22:3397-3405 and Pavletich and Pabo, (1993) Science 261:1701-1707. The incorporation of such fingers into DNA binding molecules according to the invention is envisaged.

The invention provides a solution to a problem hitherto unaddressed in the art, by permitting the rational design of polypeptides which will bind DNA triplets containing a 5-meC residue, but not identical triplets containing a C residue.

The present invention may be integrated with the rules set forth for zinc finger polypeptide design in our copending UK patent applications listed above. In a preferred aspect, therefore, the invention provides a method for preparing a DNA binding polypeptide of the Cys2-His2 zinc finger class capable of binding to a DNA triplet in target DNA sequence comprising 5-meC, but not to an identical triplet comprising unmethylated C, wherein binding to each base of the triplet by an α-helical zinc finger DNA binding motif in the polypeptide is determined as follows:

a) if the 5' base in the triplet is G, then position +6 in the α-helix is Arg and/or position ++2 is Asp;

b) if the 5' base in the triplet is A, then position +6 in the α-helix is Gln or Glu and ++2 is not Asp;

c) if the 5' base in the triplet is T, then position +6 in the α-helix is Ser or Thr and position ++2 is Asp; or position +6 is a hydrophobic amino acid other than Ala;

d) if the 5' base in the triplet is C, then position +6 in the α-helix may be any amino acid, provided that position + +2 in the α-helix is not Asp;

e) if the central base in the triplet is G, then position +3 in the α-helix is His;

f) if the central base in the triplet is A, then position +3 in the α-helix is Asn;

g) if the central base in the triplet is T, then position +3 in the α-helix is Ala, Ser, Ile, Leu, Thr or Val; provided that if it is Ala, then one of the residues at -1 or +6 is a small residue;

h) if the central base in the triplet is 5-meC, then position +3 in the α-helix is Ala;

i) if the 3' base in the triplet is G, then position -1 in the α-helix is Arg;

j) if the 3' base in the triplet is A, then position -1 in the α-helix is Gln and position +2 is Ala;

k) if the 3' base in the triplet is T, then position -1 in the α-helix is Asn; or position -1 is Gln and position +2 is Ser;

l) if the 3' base in the triplet is C, then position -1 in the α-helix is Asp and Position +1 is Arg.

The foregoing represents a set of rules which permits the design of a zinc finger binding protein specific for any given DNA sequence incorporating 5-meC.

A zinc finger binding motif is a structure well known to those in the art and defined in, for example, Miller *et al.*, (1985) EMBO J. 4:1609-1614; Berg (1988) PNAS (USA) 85:99-102; Lee *et al.*, (1989) Science 245:635-637; see International patent applications WO 96/06166 and WO 96/32475, corresponding to USSN 08/422,107, incorporated herein by reference.

In general, a preferred zinc finger framework has the structure:

(A) $X_{0-2}\ C\ X_{1-5}\ C\ X_{9-14}\ H\ X_{3-6}\ {}^{H}/_{C}$

where X is any amino acid, and the numbers in subscript indicate the possible numbers of residues represented by X.

In a preferred aspect of the present invention, zinc finger nucleic acid binding motifs may be represented as motifs having the following primary structure:

(B) X^a **C** X_{2-4} **C** X_{2-3} **F** X^c X X X X **L** X X **H** X X X^b **H** - linker

-1 1 2 3 4 5 6 7 8 9

wherein X (including X^a, X^b and X^c) is any amino acid. X_{2-4} and X_{2-3} refer to the presence of 2 or 4, or 2 or 3, amino acids, respectively. The Cys and His residues, which together co-ordinate the zinc metal atom, are marked in bold text and are usually invariant, as is the Leu residue at position +4 in the α-helix.

Modifications to this representation may occur or be effected without necessarily abolishing zinc finger function, by insertion, mutation or deletion of amino acids. For example it is known that the second His residue may be replaced by Cys (Krizek *et al.*, (1991) J. Am. Chem. Soc. 113:4518-4523) and that Leu at +4 can in some circumstances be replaced with Arg. The Phe residue before X_c may be replaced by any aromatic other than Trp. Moreover, experiments have shown that departure from the preferred structure and residue assignments for the zinc finger are tolerated and may even prove beneficial in binding to certain nucleic acid sequences. Even taking this into account, however, the general structure involving an α-helix co-ordinated by a zinc atom which contacts four Cys or His residues, does not alter. As used herein, structures (A) and (B) above are taken as an exemplary structure representing all zinc finger structures of the Cys2-His2 type.

Preferably, X^a is $^F/_Y$-X or P-$^F/_Y$-X. In this context, X is any amino acid. Preferably, in this context X is E, K, T or S. Less preferred but also envisaged are Q, V, A and P. The remaining amino acids remain possible.

Preferably, X_{2-4} consists of two amino acids rather than four. The first of these amino acids may be any amino acid, but S, E, K, T, P and R are preferred. Advantageously,

it is P or R. The second of these amino acids is preferably E, although any amino acid may be used.

Preferably, X^b is T or I.

Preferably, X^c is S or T.

Preferably, X_{2-3} is G-K-A, G-K-C, G-K-S or G-K-G. However, departures from the preferred residues are possible, for example in the form of M-R-N or M-R.

Preferably, the linker is T-G-E-K or T-G-E-K-P.

As set out above, the major binding interactions occur with amino acids -1, +3 and +6. Amino acids +4 and +7 are largely invariant. The remaining amino acids may be essentially any amino acids. Preferably, position +9 is occupied by Arg or Lys. Advantageously, positions +1, +5 and +8 are not hydrophobic amino acids, that is to say are not Phe, Trp or Tyr. Preferably, position ++2 is any amino acid, and preferably serine, save where its nature is dictated by its role as a ++2 amino acid for an N-terminal zinc finger in the same nucleic acid binding molecule.

In a most preferred aspect, therefore, bringing together the above, the invention allows the definition of every residue in a zinc finger DNA binding motif which will bind specifically to a given DNA triplet incorporating a 5-meC residue as the central residue in the triplet. Where targeting of a 5-meC containing sequence is desired, therefore, a suitable zinc finger can be constructed selecting a binding site such that 5-meC occurs at the centre of at least one base triplet thereof.

The code provided by the present invention is not entirely rigid; certain choices are provided. For example, positions +1, +5 and +8 may have any amino acid allocation, whilst other positions may have certain options: for example, the present rules provide that, for binding to a central T residue, any one of Ala, Ser or Val may

be used at +3. In its broadest sense, therefore, the present invention provides a very large number of proteins which are capable of binding to every defined target DNA triplet incorporating 5-meC as the central residue and thereby any DNA binding site incorporating 5-meC.

Preferably, however, the number of possibilities may be significantly reduced. For example, the non-critical residues +1, +5 and +8 may be occupied by the residues Lys, Thr and Gln respectively as a default option. In the case of the other choices, for example, the first-given option may be employed as a default. Thus, the code according to the present invention allows the design of a single, defined polypeptide (a "default" polypeptide) which will bind to its target triplet.

In a further aspect of the present invention, there is provided a method for preparing a DNA binding protein of the Cys2-His2 zinc finger class capable of binding to a target DNA sequence incorporating 5-meC, comprising the steps of:

a) selecting a model zinc finger domain from the group consisting of naturally occurring zinc fingers and consensus zinc fingers; and

b) mutating at least one of positions -1, +3, +6 (and ++2) of the finger as required by a method according to the present invention.

In general, naturally occurring zinc fingers may be selected from those fingers for which the DNA binding specificity is known. For example, these may be the fingers for which a crystal structure has been resolved: namely Zif 268 (Elrod-Erickson *et al.*, (1996) Structure 4:1171-1180), GLI (Pavletich and Pabo, (1993) Science 261:1701-1707), Tramtrack (Fairall *et al.*, (1993) Nature 366:483-487) and YY1 (Houbaviy *et al.*, (1996) PNAS (USA) 93:13577-13582).

The naturally occurring zinc finger 2 in Zif 268 makes an excellent starting point from which to engineer a zinc finger and is preferred.

Consensus zinc finger structures may be prepared by comparing the sequences of known zinc fingers, irrespective of whether their binding domain is known. Preferably, the consensus structure is selected from the group consisting of the consensus structure P Y K C P E C G K S F S Q K S D L V K H Q R T H T G, and the consensus structure P Y K C S E C G K A F S Q K S N L T R H Q R I H T G E K P.

The consensuses are derived from the consensus provided by Krizek *et al.*, (1991) J. Am. Chem. Soc. 113:4518-4523 and from Jacobs, (1993) PhD thesis, University of Cambridge, UK. In both cases, the linker sequences described above for joining two zinc finger motifs together, namely TGEK or TGEKP can be formed on the ends of the consensus. Thus, a P may be removed where necessary, or, in the case of the consensus terminating T G, E K (P) can be added.

When the nucleic acid specificity of the model finger selected is known, the mutation of the finger in order to modify its specificity to bind to the target DNA may be directed to residues known to affect binding to bases at which the natural and desired targets differ. Otherwise, mutation of the model fingers should be concentrated upon residues -1, +3, +6 and ++2 as provided for in the foregoing rules.

In order to produce a binding protein having improved binding, moreover, the rules provided by the present invention may be supplemented by physical or virtual modelling of the protein/DNA interface in order to assist in residue selection.

In a second embodiment, the invention provides a method for producing a zinc finger polypeptide capable of binding to a DNA sequence comprising a modified residue, but not to an identical sequence comprising an equivalent unmodified residue, comprising the steps of:

a) providing a nucleic acid library encoding a repertoire of zinc finger polypeptides, the nucleic acid members of the library being at least partially randomised

at one or more of the positions encoding residues -1, 3, 6 and ++2 of the α-helix of the zinc finger polypeptides;

b) displaying the library in a selection system and screening it against a target DNA sequence comprising the modified residue;

c) isolating the nucleic acid members of the library encoding zinc finger polypeptides capable of binding to the target sequence; and

d) optionally, verifying that the zinc finger polypeptides do not bind significantly to a DNA sequence identical to the target DNA sequence but containing the unmodified residue in place of the modified residue.

Methods for the production of libraries encoding randomised polypeptides are known in the art and may be applied in the present invention. Randomisation may be total, or partial; in the case of partial randomisation, the selected codons preferably encode options for amino acids as set forth in the rules of the first embodiment of the present invention. Thus, the first and second embodiments may advantageously be combined.

Preferably, the modified residue is 5-meC and the unmodified residue is C. However, other modifications may be targeted by the method of the invention. For example, zinc finger polypeptides may be designed which specifically bind to nucleic acids incorporating the base U, in preference to the equivalent base T. An advantage of the second embodiment of the invention is that zinc finger polypeptides may be developed to bind to any DNA sequence incorporating a modified base, irrespective of its positioning in the target DNA triplet.

Randomisation involves may involve of zinc finger polypeptides at the DNA or protein level. Mutagenesis and screening of zinc finger polypeptides may be achieved by any suitable means. Preferably, the mutagenesis is performed at the nucleic acid level, for example by synthesising novel genes encoding mutant proteins and expressing these to

obtain a variety of different proteins. Alternatively, existing genes can be themselves mutated, such by site-directed or random mutagenesis, in order to obtain the desired mutant genes.

Mutations may be performed by any method known to those of skill in the art. Preferred, however, is site-directed mutagenesis of a nucleic acid sequence encoding the protein of interest. A number of methods for site-directed mutagenesis are known in the art, from methods employing single-stranded phage such as M13 to PCR-based techniques (see "PCR Protocols: A guide to methods and applications", M.A. Innis, D.H. Gelfand, J.J. Sninsky, T.J. White (eds.). Academic Press, New York, 1990). Preferably, the commercially available Altered Site II Mutagenesis System (Promega) may be employed, according to the directions given by the manufacturer.

Randomisation of the zinc finger binding motifs produced according to the invention is preferably directed to those residues where the code provided herein gives a choice of residues. For example, therefore, positions +1, +5 and +8 are advantageously randomised, whilst preferably avoiding hydrophobic amino acids; positions involved in binding to the nucleic acid, notably -1, +2, +3 and +6, may be randomised also, preferably within the choices provided by the rules of the present invention.

Screening of the proteins produced by mutant genes is preferably performed by expressing the genes and assaying the binding ability of the protein product. A simple and advantageously rapid method by which this may be accomplished is by phage display, in which the mutant polypeptides are expressed as fusion proteins with the coat proteins of filamentous bacteriophage, such as the minor coat protein pII of bacteriophage m13 or gene III of bacteriophage Fd, and displayed on the capsid of bacteriophage transformed with the mutant genes. The target nucleic acid sequence is used as a probe to bind directly to the protein on the phage surface and select the phage possessing advantageous mutants, by affinity purification. The phage are then amplified by passage through a bacterial host, and subjected to further rounds of selection and amplification in order to enrich the mutant pool for the desired phage and

eventually isolate the preferred clone(s). Detailed methodology for phage display is known in the art and set forth, for example, in US Patent 5,223,409; Choo and Klug, (1995) Current Opinions in Biotechnology 6:431-436; Smith, (1985) Science 228:1315-1317; and McCafferty *et al.*, (1990) Nature 348:552-554; all incorporated herein by reference. Vector systems and kits for phage display are available commercially, for example from Pharmacia.

Specific peptide ligands such as zinc finger polypeptides may moreover be selected for binding to targets by affinity selection using large libraries of peptides linked to the C terminus of the lac-repressor-LacI (Cull *et al.*, (1992) Proc Natl Acad Sci U S A, 89, 1865-9). When expressed in *E. coli* the repressor protein physically links the ligand to the encoding plasmid by binding to a lac operator sequence on the plasmid.

An entirely *in vitro* polysome display system has also been reported (Mattheakis *et al.*, (1994) Proc Natl Acad Sci U S A, 91, 9022-6) in which nascent peptides are physically attached via the ribosome to the RNA which encodes them.

The library of the invention may randomised at those positions for which choices are given in the rules of the first embodiment of the present invention. In particular, the members of the library are randomised at position+3 for binding to a central 5-meC residue. In such a case, 5-meC binding polypeptides will be selected by comparative binding analyses against methylated and non-methylated binding sites. However, the rules set forth above allow the person of ordinary skill in the art to make informed choices concerning the desired codon usage at the given positions. For instance, position +3 in the case of a central 5-meC residue should be Ala residue, encoded by the codon GCN.

In a third embodiment, the present invention may be applied to the production of zinc finger polypeptides capable of binding to a DNA sequence comprising an unmethylated C residue, but not to an identical sequence comprising a 5-meC residue. This may be

carried out by differential screening, as set forth above. Moreover, rules may be applied in addition to or instead of screening.

Where the central residue of a target triplet is C, the use of Asp at position +3 of a zinc finger polypeptide allows preferential binding to C over 5-meC.

Zinc finger binding motifs designed according to the invention may be combined into nucleic acid binding proteins having a multiplicity of zinc fingers. Preferably, the proteins have at least two zinc fingers. In nature, zinc finger binding proteins commonly have at least three zinc fingers, although two zinc finger proteins such as Tramtrack are known. The presence of at least three zinc fingers is preferred. Binding proteins may be constructed by joining the required fingers end to end, N-terminus to C-terminus. Preferably, this is effected by joining together the relevant nucleic acid coding sequences encoding the zinc fingers to produce a composite coding sequence encoding the entire binding protein. The invention therefore provides a method for producing a DNA binding protein as defined above, wherein the DNA binding protein is constructed by recombinant DNA technology, the method comprising the steps of:

a) preparing a nucleic acid coding sequence encoding two or more zinc finger binding motifs as defined above, placed N-terminus to C-terminus;
b) inserting the nucleic acid sequence into a suitable expression vector; and
c) expressing the nucleic acid sequence in a host organism in order to obtain the DNA binding protein.

A "leader" peptide may be added to the N-terminal finger. Preferably, the leader peptide is MAEEKP.

The nucleic acid encoding the DNA binding protein according to the invention can be incorporated into vectors for further manipulation. As used herein, vector (or plasmid) refers to discrete elements that are used to introduce heterologous nucleic acid into cells for either expression or replication thereof. Selection and use of such vehicles are well

within the skill of the person of ordinary skill in the art. Many vectors are available, and selection of appropriate vector will depend on the intended use of the vector, i.e. whether it is to be used for DNA amplification or for nucleic acid expression, the size of the DNA to be inserted into the vector, and the host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell for which it is compatible. The vector components generally include, but are not limited to, one or more of the following: an origin of replication, one or more marker genes, an enhancer element, a promoter, a transcription termination sequence and a signal sequence.

Both expression and cloning vectors generally contain nucleic acid sequence that enable the vector to replicate in one or more selected host cells. Typically in cloning vectors, this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (e.g. SV 40, polyoma, adenovirus) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors unless these are used in mammalian cells competent for high level DNA replication, such as COS cells.

Most expression vectors are shuttle vectors, i.e. they are capable of replication in at least one class of organisms but can be transfected into another class of organisms for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells even though it is not capable of replicating independently of the host cell chromosome. DNA may also be replicated by insertion into the host genome. However, the recovery of genomic DNA encoding the DNA binding protein is more complex than that of exogenously replicated vector because restriction enzyme digestion is required to excise DNA binding protein DNA. DNA can

be amplified by PCR and be directly transfected into the host cells without any replication component.

Advantageously, an expression and cloning vector may contain a selection gene also referred to as selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that confer resistance to antibiotics and other toxins, e.g. ampicillin, neomycin, methotrexate or tetracycline, complement auxotrophic deficiencies, or supply critical nutrients not available from complex media.

As to a selective gene marker appropriate for yeast, any marker gene can be used which facilitates the selection for transformants due to the phenotypic expression of the marker gene. Suitable markers for yeast are, for example, those conferring resistance to antibiotics G418, hygromycin or bleomycin, or provide for prototrophy in an auxotrophic yeast mutant, for example the URA3, LEU2, LYS2, TRP1, or HIS3 gene.

Since the replication of vectors is conveniently done in *E. coli*, an *E. coli* genetic marker and an *E. coli* origin of replication are advantageously included. These can be obtained from *E. coli* plasmids, such as pBR322, Bluescript© vector or a pUC plasmid, e.g. pUC18 or pUC19, which contain both *E. coli* replication origin and *E. coli* genetic marker conferring resistance to antibiotics, such as ampicillin.

Suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up DNA binding protein nucleic acid, such as dihydrofolate reductase (DHFR, methotrexate resistance), thymidine kinase, or genes conferring resistance to G418 or hygromycin. The mammalian cell transformants are placed under selection pressure which only those transformants which have taken up and are expressing the marker are uniquely adapted to survive. In the case of a DHFR or glutamine synthase (GS) marker, selection pressure can be imposed by culturing the transformants under conditions in which the pressure is progressively increased,

thereby leading to amplification (at its chromosomal integration site) of both the selection gene and the linked DNA that encodes the DNA binding protein. Amplification is the process by which genes in greater demand for the production of a protein critical for growth, together with closely associated genes which may encode a desired protein, are reiterated in tandem within the chromosomes of recombinant cells. Increased quantities of desired protein are usually synthesised from thus amplified DNA.

Expression and cloning vectors usually contain a promoter that is recognised by the host organism and is operably linked to DNA binding protein encoding nucleic acid. Such a promoter may be inducible or constitutive. The promoters are operably linked to DNA encoding the DNA binding protein by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native DNA binding protein promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of DNA binding protein encoding DNA.

Promoters suitable for use with prokaryotic hosts include, for example, the β-lactamase and lactose promoter systems, alkaline phosphatase, the tryptophan (trp) promoter system and hybrid promoters such as the tac promoter. Their nucleotide sequences have been published, thereby enabling the skilled worker operably to ligate them to DNA encoding DNA binding protein, using linkers or adapters to supply any required restriction sites. Promoters for use in bacterial systems will also generally contain a Shine-Delgarno sequence operably linked to the DNA encoding the DNA binding protein.

Preferred expression vectors are bacterial expression vectors which comprise a promoter of a bacteriophage such as phagex or T7 which is capable of functioning in the bacteria. In one of the most widely used expression systems, the nucleic acid encoding the fusion protein may be transcribed from the vector by T7 RNA polymerase (Studier et al, Methods in Enzymol. 185; 60-89, 1990). In the *E. coli* BL21(DE3)

host strain, used in conjunction with pET vectors, the T7 RNA polymerase is produced from the λ-lysogen DE3 in the host bacterium, and its expression is under the control of the IPTG inducible lac UV5 promoter. This system has been employed successfully for over-production of many proteins. Alternatively the polymerase gene may be introduced on a lambda phage by infection with an int- phage such as the CE6 phage which is commercially available (Novagen, Madison, USA). other vectors include vectors containing the lambda PL promoter such as PLEX (Invitrogen, NL) , vectors containing the trc promoters such as pTrcHisXpressTm (Invitrogen) or pTrc99 (Pharmacia Biotech, SE) or vectors containing the tac promoter such as pKK223-3 (Pharmacia Biotech) or PMAL (New England Biolabs, MA, USA).

Moreover, the DNA binding protein gene according to the invention preferably includes a secretion sequence in order to facilitate secretion of the polypeptide from bacterial hosts, such that it will be produced as a soluble native peptide rather than in an inclusion body. The peptide may be recovered from the bacterial periplasmic space, or the culture medium, as appropriate.

Suitable promoting sequences for use with yeast hosts may be regulated or constitutive and are preferably derived from a highly expressed yeast gene, especially a Saccharomyces cerevisiae gene. Thus, the promoter of the TRP1 gene, the ADHI or ADHII gene, the acid phosphatase (PH05) gene, a promoter of the yeast mating pheromone genes coding for the a- or α-factor or a promoter derived from a gene encoding a glycolytic enzyme such as the promoter of the enolase, glyceraldehyde-3-phosphate dehydrogenase (GAP), 3-phospho glycerate kinase (PGK), hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triose phosphate isomerase, phosphoglucose isomerase or glucokinase genes, or a promoter from the TATA binding protein (TBP) gene can be used. Furthermore, it is possible to use hybrid promoters comprising upstream activation sequences (UAS) of one yeast gene and downstream promoter elements including a functional TATA box of another yeast gene, for example a hybrid promoter including the UAS(s) of the yeast PH05 gene and downstream

promoter elements including a functional TATA box of the yeast GAP gene (PH05-GAP hybrid promoter). A suitable constitutive PHO5 promoter is e.g. a shortened acid phosphatase PH05 promoter devoid of the upstream regulatory elements (UAS) such as the PH05 (-173) promoter element starting at nucleotide -173 and ending at nucleotide -9 of the PH05 gene.

DNA binding protein gene transcription from vectors in mammalian hosts may be controlled by promoters derived from the genomes of viruses such as polyoma virus, adenovirus, fowlpox virus, bovine papilloma virus, avian sarcoma virus, cytomegalovirus (CMV), a retrovirus and Simian Virus 40 (SV40), from heterologous mammalian promoters such as the actin promoter or a very strong promoter, e.g. a ribosomal protein promoter, and from the promoter normally associated with DNA binding protein sequence, provided such promoters are compatible with the host cell systems.

Transcription of a DNA encoding DNA binding protein by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are relatively orientation and position independent. Many enhancer sequences are known from mammalian genes (e.g. elastase and globin). However, typically one will employ an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270) and the CMV early promoter enhancer. The enhancer may be spliced into the vector at a position 5' or 3' to DNA binding protein DNA, but is preferably located at a site 5' from the promoter.

Advantageously, a eukaryotic expression vector encoding a DNA binding protein according to the invention may comprise a locus control region (LCR). LCRs are capable of directing high-level integration site independent expression of transgenes integrated into host cell chromatin, which is of importance especially where the DNA binding protein gene is to be expressed in the context of a permanently-transfected eukaryotic cell line in which chromosomal integration of the vector has occurred, or in transgenic animals.

Eukaryotic vectors may also contain sequences necessary for the termination of transcription and for stabilising the mRNA. Such sequences are commonly available from the 5' and 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding DNA binding protein.

An expression vector includes any vector capable of expressing DNA binding protein nucleic acids that are operatively linked with regulatory sequences, such as promoter regions, that are capable of expression of such DNAs. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector, that upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those with ordinary skill in the art and include those that are replicable in eukaryotic and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome. For example, DNAs encoding DNA binding protein may be inserted into a vector suitable for expression of cDNAs in mammalian cells, e.g. a CMV enhancer-based vector such as pEVRF (Matthias, et al., (1989) NAR 17, 6418).

Particularly useful for practising the present invention are expression vectors that provide for the transient expression of DNA encoding DNA binding protein in mammalian cells. Transient expression usually involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector, and, in turn, synthesises high levels of DNA binding protein. For the purposes of the present invention, transient expression systems are useful e.g. for identifying DNA binding protein mutants, to identify potential phosphorylation sites, or to characterise functional domains of the protein.

Construction of vectors according to the invention employs conventional ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in

the form desired to generate the plasmids required. If desired, analysis to confirm correct sequences in the constructed plasmids is performed in a known fashion. Suitable methods for constructing expression vectors, preparing in vitro transcripts, introducing DNA into host cells, and performing analyses for assessing DNA binding protein expression and function are known to those skilled in the art. Gene presence, amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA, dot blotting (DNA or RNA analysis), or in situ hybridisation, using an appropriately labelled probe which may be based on a sequence provided herein. Those skilled in the art will readily envisage how these methods may be modified, if desired.

In accordance with another embodiment of the present invention, there are provided cells containing the above-described nucleic acids. Such host cells such as prokaryote, yeast and higher eukaryote cells may be used for replicating DNA and producing the DNA binding protein. Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, such as *E. coli*, e.g. *E. coli* K-12 strains, DH5a and HB101, or Bacilli. Further hosts suitable for the DNA binding protein encoding vectors include eukaryotic microbes such as filamentous fungi or yeast, e.g. Saccharomyces cerevisiae. Higher eukaryotic cells include insect and vertebrate cells, particularly mammalian cells including human cells, or nucleated cells from other multicellular organisms. In recent years propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are epithelial or fibroblastic cell lines such as Chinese hamster ovary (CHO) cells, NIH 3T3 cells, HeLa cells or 293T cells. The host cells referred to in this disclosure comprise cells in *in vitro* culture as well as cells that are within a host animal.

DNA may be stably incorporated into cells or may be transiently expressed using methods known in the art. Stably transfected mammalian cells may be prepared by transfecting cells with an expression vector having a selectable marker gene, and growing the transfected cells under conditions selective for cells expressing the marker

gene. To prepare transient transfectants, mammalian cells are transfected with a reporter gene to monitor transfection efficiency.

To produce such stably or transiently transfected cells, the cells should be transfected with a sufficient amount of the DNA binding protein-encoding nucleic acid to form the DNA binding protein. The precise amounts of DNA encoding the DNA binding protein may be empirically determined and optimised for a particular cell and assay.

Host cells are transfected or, preferably, transformed with the above-captioned expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. Heterologous DNA may be introduced into host cells by any method known in the art, such as transfection with a vector encoding a heterologous DNA by the calcium phosphate coprecipitation technique or by electroporation. Numerous methods of transfection are known to the skilled worker in the field. Successful transfection is generally recognised when any indication of the operation of this vector occurs in the host cell. Transformation is achieved using standard techniques appropriate to the particular host cells used.

Incorporation of cloned DNA into a suitable expression vector, transfection of eukaryotic cells with a plasmid vector or a combination of plasmid vectors, each encoding one or more distinct genes or with linear DNA, and selection of transfected cells are well known in the art (see, e.g. Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press).

Transfected or transformed cells are cultured using media and culturing methods known in the art, preferably under conditions, whereby the DNA binding protein encoded by the DNA is expressed. The composition of suitable media is known to those in the art, so that they can be readily prepared. Suitable culturing media are also commercially available.

DNA binding proteins according to the invention may be employed in a wide variety of applications, including diagnostics and as research tools. Advantageously, they may be employed as diagnostic tools for identifying the presence of modified nucleic acid molecules in a complex mixture. DNA binding molecules according to the invention can differentiate single base modifications in target DNA molecules.

5-meC targeting zinc fingers may moreover be employed in the regulation of gene transcription, for example by specific cleavage of methylated (or unmethylated) sequences using a fusion polypeptide comprising a zinc finger targeting domain and a DNA cleavage domain. Zinc fingers capable of differentiating between U and T may be used to preferentially target RNA or DNA, as required. Where RNA-targeting polypeptides are intended, these are included in the term "DNA-binding molecule".

In a preferred embodiment, the zinc finger polypeptides of the invention may be employed to detect the presence of a particular base modification in a target nucleic acid sequence in a sample.

Accordingly, the invention provides a method for determining the presence of a target modified nucleic acid molecule, comprising the steps of:

a) preparing a DNA binding protein by the method set forth above which is specific for the target modified nucleic acid molecule;
b) exposing a test system comprising the target modified nucleic acid molecule to the DNA binding protein under conditions which promote binding, and removing any DNA binding protein which remains unbound;
c) detecting the presence of the DNA binding protein in the test system.

In a preferred embodiment, the DNA binding molecules of the invention can be incorporated into an ELISA assay. For example, phage displaying the molecules of the invention can be used to detect the presence of the target DNA, and visualised using enzyme-linked anti-phage antibodies.

Further improvements to the use of zinc finger phage for diagnosis can be made, fo example, by co-expressing a marker protein fused to the minor coat protein (gVIII) of bacteriophage. Since detection with an anti-phage antibody would then be obsolete, the time and cost of each diagnosis would be further reduced. Depending on the requirements, suitable markers for display might include the fluorescent proteins (A. B. Cubitt, *et al.*, (1995) *Trends Biochem Sci.* **20**, 448-455; T. T. Yang, *et al.*, (1996) *Gene* **173**, 19-23), or an enzyme such as alkaline phosphatase which has been previously displayed on gIII (J. McCafferty, R. H. Jackson, D. J. Chiswell, (1991) *Protein Engineering* **4**, 955-961) Labelling different types of diagnostic phage with distinct markers would allow multiplex screening of a single DNA sample. Nevertheless, even in the absence of such refinements, the basic ELISA technique is reliable, fast, simple and particularly inexpensive. Moreover it requires no specialised apparatus, nor does it employ hazardous reagents such as radioactive isotopes, making it amenable to routine use in the clinic. The major advantage of the protocol is that it obviates the requirement for gel electrophoresis, and so opens the way to automated DNA diagnosis.

The invention provides DNA binding proteins which can be engineered with exquisite specificity. The invention lends itself, therefore, to the design of any molecule of which specific DNA binding is required. For example, the proteins according to the invention may be employed in the manufacture of chimeric restriction enzymes, in which a nucleic acid cleaving domain is fused to a DNA binding domain comprising a zinc finger as described herein.

The invention is described below, for the purpose of illustration only, in the following examples, with reference to the figures, in which:

Figure 1a is an alignment of the amino acid sequence of the three fingers from Zif268 used in a phage display library. Randomised residue positions in the α-helix of finger 2 are marked 'X' and are numbered above the alignment relative to the first helical

residue (position +1). Residues which form the hydrophobic core are circled; zinc ligands are written as white letters on a black circle background; and positions comprising the secondary structure elements of a zinc finger are marked below the sequence.

Figure 1b shows amino acid sequences of the variant α-helical regions from some zinc fingers selected by phage display using the DNA binding site GCGGNGGCG where the central (bold) nucleotide of the middle (underlined) triplet was either: (i) 5-methylcytosine, (ii) thymine, or (iii) cytosine. Amino acid sequences are listed below the DNA oligonucleotide used in their selection. Amino acid positions are numbered above the aligned sequences relative to the first helical residue (position +1). Circled residues (in position +3) are predicted to contact the middle nucleotide of the binding site.

Figure 1c shows a phage ELISA binding assay showing discrimination of pyrimidines by representative phage-selected zinc fingers. The matrix shows three different zinc finger phage clones (x, y and z) reacted with four different DNA binding sites present at a concentration of 3nM. Binding is represented by vertical bars which indicate the OD obtained by ELISA (Choo and Klug, (1997) Curr. Opin. Str. Biol. 7:117-125). The amino acid sequences of the variant α-helical regions from the selected zinc fingers are: REDVLIRHGK (x), RADALMVHKR (y), and RGPDLARHGR (z). The DNA sequences contain the generic binding site GCGGNGGCG, where the central (bold) nucleotide was either: uracil (U), thymine (T), cytosine (C), or 5-methylcytosine (M).

Figure 2 shows the effect of cytosine methylation on DNA binding by phage-selected zinc fingers. Graphs show three different zinc finger phage binding to the DNA sequence GCGGCGGCG in the presence (circle) and absence (triangle) of methylation of the central base (bold). The zinc finger clones tested contained variant α-helical regions of the middle finger as follows: (a) RADALMVHKR, (b) RGPDLARHGR and (c) REDVLIRHGK. These respective zinc finger clones preferentially bind their cognate DNA site in the presence, absence, or regardless of cytosine methylation.

Example 1

Preparation and Screening of a Zinc Finger Phage Display Library

A powerful method of selecting DNA binding proteins is the cloning of peptides (Smith (1985) Science 228, 1315-1317), or protein domains (McCafferty *et al.*, (1990) Nature 348:552-554; Bass *et al.*, (1990) Proteins 8:309-314), as fusions to the minor coat protein (pIII) of bacteriophage fd, which leads to their expression on the tip of the capsid. A phage display library is created comprising variants of the middle finger from the DNA binding domain of Zif268.

Materials And Methods

Construction And Cloning Of Genes. In general, procedures and materials are in accordance with guidance given in Sambrook *et al.*, Molecular Cloning. A Laboratory Manual, Cold Spring Harbor, 1989. The gene for the Zif268 fingers (residues 333-420) is assembled from 8 overlapping synthetic oligonucleotides (see Choo and Klug, (1994) PNAS (USA) 91:11163-67), giving *Sfi*I and *Not*I overhangs. The genes for fingers of the phage library are synthesised from 4 oligonucleotides by directional end to end ligation using 3 short complementary linkers, and amplified by PCR from the single strand using forward and backward primers which contain sites for *Not*I and *Sfi*I respectively. Backward PCR primers in addition introduce Met-Ala-Glu as the first three amino acids of the zinc finger peptides, and these are followed by the residues of the wild type or library fingers as required. Cloning overhangs are produced by digestion with *Sfi*I and *Not*I where necessary. Fragments are ligated to 1µg similarly prepared Fd-Tet-SN vector. This is a derivative of fd-tet-DOG1 (Hoogenboom *et al.*, (1991) Nucleic Acids Res. 19, 4133-4137) in which a section of the pelB leader and a restriction site for the enzyme *Sfi*I (underlined) have been added by site-directed mutagenesis using the oligonucleotide:

5' CTCCTGCAGTTGGACCTGTGCCATGGCCGGCTGGGCCGCATAGAATGG AACAACTAAAGC 3' (Seq ID No. 1)

which anneals in the region of the polylinker. Electrocompetent DH5α cells are transformed with recombinant vector in 200ng aliquots, grown for 1 hour in 2xTY medium with 1% glucose, and plated on TYE containing 15μg/ml tetracycline and 1% glucose.

Figure 1 shows the amino acid sequences of the three zinc fingers derived from Zif268 used in the phage display library of the present invention. The top and bottom rows represent the sequence of the first and third fingers respectively. The middle row represents the sequence of the middle finger. The randomised positions in the α-helix of the middle finger have residues marked 'X'. The amino acid positions are numbered relative to the first helical residue (position 1). For amino acids at positions -1 to +8, excluding the conserved Leu and His, codons are equal mixtures of (G,A,C)NN: T in the first base position is omitted in order to avoid stop codons, but this has the unfortunate effect that the codons for Trp, Phe, Tyr and Cys are not represented. Position +9 is specified by the codon A(G,A)G, allowing either Arg or Lys. Residues of the hydrophobic core are circled, whereas the zinc ligands are written as white letters on black circles. The positions forming the β-sheets and the α-helix of the zinc fingers are marked below the sequence.

Phage Selection. Colonies are transferred from plates to 200ml 2xTY/Zn/Tet (2xTY containing 50μM $Zn(CH_3COO)_2$ and 15μg/ml tetracycline) and grown overnight. Phage are purified from the culture supernatant by two rounds of precipitation using 0.2 volumes of 20% PEG/2.5M NaCl containing 50μM $Zn(CH3.COO)_2$, and resuspended in zinc finger phage buffer (20mM HEPES pH7.5, 50mM NaCl, 1mM $MgCl_2$ and 50μM $Zn(CH3.COO)_2$). Streptavidin-coated paramagnetic beads (Dynal) are washed in zinc finger phage buffer and blocked for 1 hour at room temperature with the same buffer made up to 6% in fat-free dried milk (Marvel). Selection of phage is over three rounds: in the first round, beads (1 mg) are saturated with biotinylated oligonucleotide (~80nM) and then washed prior to phage binding, but in the second and third rounds 1.7nM oligonucleotide and 5μg poly dGC (Sigma) are added to the beads with the phage.

Binding reactions (1.5ml) for 1 hour at 15°C are in zinc finger phage buffer made up to 2% in fat-free dried milk (Marvel) and 1% in Tween 20, and typically contained $5x10^{11}$ phage. Beads are washed 15 times with 1ml of the same buffer. Phage are eluted by shaking in 0.1M triethylamine for 5min and neutralised with an equal volume of 1M Tris pH7.4. Log phase *E. coli* TG1 in 2xTY are infected with eluted phage for 30min at 37°C and plated as described above. Phage titres are determined by plating serial dilutions of the infected bacteria.

Sequencing Of Selected Phage. Single colonies of transformants obtained after three rounds of selection as described, are grown overnight in 2xTY/Zn/Tet. Small aliquots of the cultures are stored in 15% glycerol at -20°C, to be used as an archive. Single-stranded DNA is prepared from phage in the culture supernatant and sequenced using the Sequenase™ 2.0 kit (U.S. Biochemical Corp.).

Example 2

Isolation of zinc fingers capable of C-T differentiation

The phage are selected against oligonucleotides comprising the sequences GCGGCGGCG and GCGGTGGCG. some zinc finger DNA-binding domains are selected which bound both sequences equally well (Fig. 1b, c). However, two additional zinc finger families are isolated which are capable of differential binding to the two closely related sites (Fig. 1b, c). Sequence-specific recognition requires discrimination of the central base in the binding site by amino acids in position 3 of the recognition helix of the selected zinc fingers, and it is noted that aspartate is selected to bind opposite cytosine in the triplet GCG, while alanine is selected opposite thymine in the triplet GTG. The correlation between thymine and alanine is particularly significant, as it implies a van der Waals interaction between the amino acid side-chain and the 5-methyl group of the base. Indeed, when thymine is mutated to deoxyuracil in the binding sites of such fingers there is a dramatic decrease in the strength of the intermolecular interaction (Fig 1c). This shows that these zinc fingers are capable of specifically recognising a 5-

methyl group, and suggests that similar fingers might be selected which bind 5-meC by the same token.

Example 3

Selection of 5-methylcysteine-specific zinc fingers

The phage display library is screened with the synthetic binding site GCGGMGGCG, containing a 5meC base analogue (M). After 5 rounds of selection, zinc finger phage are tested for binding to 5-meC and cytosine in the context of the above site, and those capable of specifically binding the methylated site are sequenced in the region of the zinc finger gene. Two different clones are isolated, which are identical to the DNA-binding domains previously selected using the binding site GCGGTGGCG.

Hence the various zinc finger phage selections described above yield different fingers able to bind the generic DNA sequence GCGGNGGCG, where N is either thymine, cytosine or 5-meC. A full complement of fingers is selected for recognition of the cytosine/5-meC pair in the above context, some of which recognise one type of base exclusively, while others bound both bases equally well (Figures 1c and 2).

The zinc finger amino acid residues which are selected by the interaction between the randomised recognition helix and the central base of the DNA binding site are rationalised in terms of previously elucidated zinc finger-DNA recognition rules. Fingers with alanine in position +3 of the recognition helix specifically bind 5-meC and thymine owing to a tight hydrophobic interaction between the side chain and the 5-methyl group which is present in both bases. In contrast, a finger with valine in position +3 is also able to accommodate cytosine in addition to the two methylated bases, by the use of different rotamers. Fingers with aspartate in position +3 bind cytosine specifically, for example by forming a ring structure which packs against the pyrimidine as is observed in the refined crystal structure of Zif268.

Claims

1. A zinc finger polypeptide which binds to a target DNA sequence containing a modified base but not to an identical sequence containing the equivalent unmodified base.

2. A polypeptide according to claim 1, wherein the target DNA sequence comprises a triplet having 5-meC at the central position, and binding to the 5-meC residue by an α-helical zinc finger binding motif in the polypeptide is achieved by placing an Ala residue at position +3 of the α-helix.

3. A method for preparing a DNA binding polypeptide of the Cys2-His2 zinc finger class capable of binding to a DNA triplet in target DNA sequence comprising 5-meC as the central residue in the target DNA triplet, wherein binding to the 5-meC residue by an α-helical zinc finger DNA binding motif of the polypeptide is achieved by placing an Ala residue at position +3 of the α-helix of the zinc finger.

4. A method for preparing a DNA binding polypeptide of the Cys2-His2 zinc finger class capable of binding to a DNA triplet in target DNA sequence comprising 5-meC, but not to an identical triplet comprising unmethylated C, wherein binding to each base of the triplet by an α-helical zinc finger DNA binding motif in the polypeptide is determined as follows:

a) if the 5' base in the triplet is G, then position +6 in the α-helix is Arg and/or position + +2 is Asp;

b) if the 5' base in the triplet is A, then position +6 in the α-helix is Gln or Glu and + +2 is not Asp;

c) if the 5' base in the triplet is T, then position +6 in the α-helix is Ser or Thr and position + +2 is Asp; or position +6 is a hydrophobic amino acid other than Ala;

d) if the 5' base in the triplet is C, then position +6 in the α-helix may be any amino acid, provided that position + +2 in the α-helix is not Asp;

e) if the central base in the triplet is G, then position +3 in the α-helix is His;

f) if the central base in the triplet is A, then position +3 in the α-helix is Asn;

g) if the central base in the triplet is T, then position +3 in the α-helix is Ala, Ser, Ile, Leu, Thr or Val; provided that if it is Ala, then one of the residues at -1 or +6 is a small residue;

h) if the central base in the triplet is 5-meC, then position +3 in the α-helix is Ala;

i) if the 3' base in the triplet is G, then position -1 in the α-helix is Arg;

j) if the 3' base in the triplet is A, then position -1 in the α-helix is Gln and position +2 is Ala;

k) if the 3' base in the triplet is T, then position -1 in the α-helix is Asn; or position -1 is Gln and position +2 is Ser;

l) if the 3' base in the triplet is C, then position -1 in the α-helix is Asp and Position +1 is Arg.

5. A method for producing a zinc finger polypeptide capable of binding to a DNA sequence comprising a modified residue, but not to an identical sequence comprising an equivalent unmodified residue, comprising the steps of:

a) providing a DNA library encoding a repertoire of zinc finger polypeptides, the DNA members of the library being at least partially randomised at one or more of the positions encoding residues -1, 3, 6 and ++2 of an α-helical zinc finger binding motif of the zinc finger polypeptides;

b) displaying the library in a selection system and screening it against a target DNA sequence comprising the modified residue;

c) isolating the DNA members of the library encoding zinc finger polypeptides capable of binding to the target sequence; and

d) optionally, verifying that the zinc finger polypeptides do not bind significantly to a DNA sequence identical to the target DNA sequence but containing the equivalent unmodified residue in place of the modified residue.

6. A method according to claim 5, wherein the modified residue is 5-meC and the unmodified residue is C.

7. A method according to claim 5, wherein the modified residue is U and the unmodified residue is T.

8. A method according to any one of claims 5 to 7, wherein the library is screened by phage display.

9. A method according to any one of claims 5 to 8, wherein the or each zinc finger has the general primary structure

(A) X^a C X_{2-4} C X_{2-3} F X^c X X X X L X X H X X X^b H - linker

-1 1 2 3 4 5 6 7 8 9

wherein X (including X^a, X^b and X^c) is any amino acid.

10. A method according to claim 9 wherein X^a is $^F/_Y$-X or P-$^F/_Y$-X.

11. A method according to claim 9 or claim 10 wherein X_{2-4} is selected from any one of: S-X, E-X, K-X, T-X, P-X and R-X.

12. A method according to any one of claims 9 to 11 wherein X^b is T or I.

13. A method according to any one of claims 9 to 12 wherein X_{2-3} is G-K-A, G-K-C, G-K-S, G-K-G, M-R-N or M-R.

14. A method according to any one of claims 9 to 13 wherein the linker is T-G-E-K or T-G-E-K-P.

15. A method according to any one of claims 9 to 14 wherein position +9 is R or K.

16. A method according to any one of claims 9 to 15 wherein positions +1, +5 and +8 are not occupied by any one of the hydrophobic amino acids, F, W or Y.

17. A method according to claim 16 wherein positions +1, +5 and +8 are occupied by the residues K, T and Q respectively.

18. A method for preparing a DNA binding polypeptide of the Cys2-His2 zinc finger class capable of binding to a DNA triplet in target DNA sequence comprising 5-meC, but not to an identical triplet comprising unmethylated C:

a) selecting a model zinc finger domain from the group consisting of naturally occurring zinc fingers and consensus zinc fingers; and

b) mutating the finger by the method of any one of claims 3 to 17.

19. A method according to claim 18, wherein the model zinc finger is a consensus zinc finger whose structure is selected from the group consisting of the consensus structure P Y K C P E C G K S F S Q K S D L V K H Q R T H T G, and the consensus structure P Y K C S E C G K A F S Q K S N L T R H Q R I H T G E K P.

20. A method according to claim 18 wherein the model zinc finger is a naturally occurring zinc finger whose structure is selected from one finger of a protein selected from the group consisting of Zif 268 (Elrod-Erickson *et al.*, (1996) Structure 4:1171-1180), GLI (Pavletich and Pabo, (1993) Science 261:1701-1707), Tramtrack (Fairall *et*

al., (1993) Nature 366:483-487) and YY1 (Houbaviy *et al.*, (1996) PNAS (USA) 93:13577-13582).

21. A method according to claim 20 wherein the model zinc finger is finger 2 of Zif 268.

22. A method according to any one of claims 3 to 21 wherein the binding protein comprises two or more zinc finger binding motifs, placed N-terminus to C-terminus.

23. A method according to claim 22, wherein the N-terminal zinc finger is preceded by a leader peptide having the sequence MAEEKP.

24. A method according to claim 22 or claim 23, wherein the DNA binding protein is constructed by recombinant DNA technology, the method comprising the steps of:

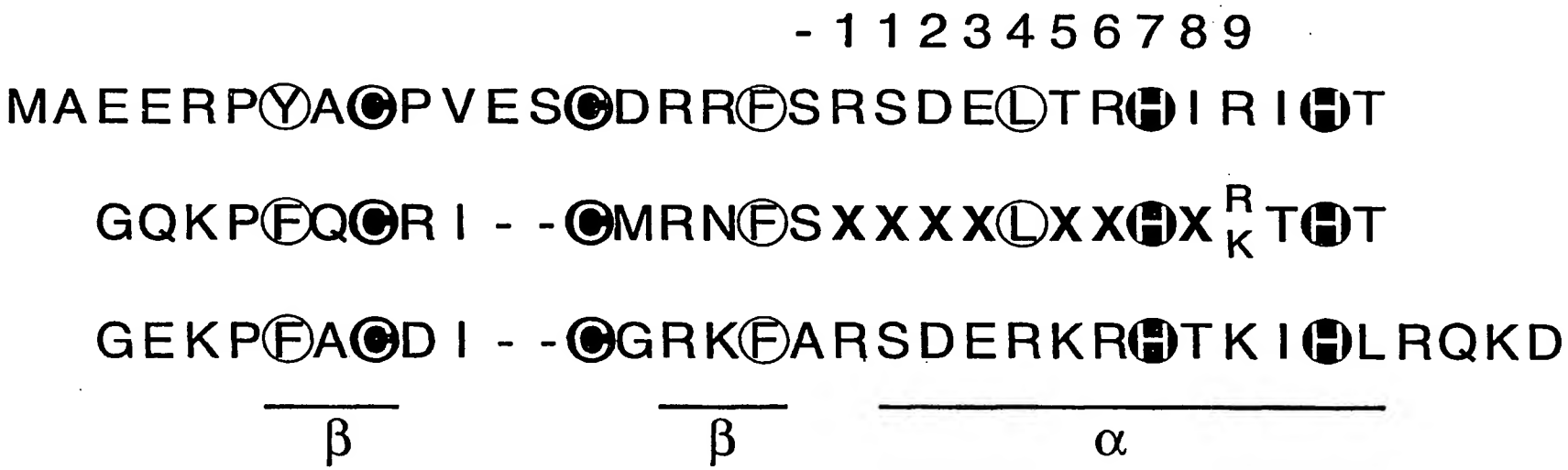
a) preparing a DNA coding sequence encoding two or more zinc finger binding preparable according to claim 23 or 24, placed N-terminus to C-terminus;
b) inserting the DNA sequence into a suitable expression vector; and
c) expressing the DNA sequence in a host organism in order to obtain the DNA binding protein.

25. A method according to one of claims 3 to 24 comprising the additional steps of subjecting the DNA binding protein to one or more rounds of randomisation and selection in order to improve the characteristics thereof.

Abstract

The invention provides a method for producing a zinc finger polypeptide which binds to a target nucleic acid sequence containing a modified base but not to an identical sequence containing an equivalent unmodified base.

Figure 1 A


-1 1 2 3 4 5 6 7 8 9
MAEERPYACPVESCDRRFSRSDELTRHIRIHT
GQKPFQCRI--CMRNFSXXXXLXXHXR/KTHT
GEKPFACDI--CGRKFARSDERKRHTKIHLRQKD
β
β
α

(i) GCGG**M**GGCG

-1123456789
RADÂLMVHKR
RGDÂLTSHER

(ii) GCGG**T**GGCG

-1123456789
RADÂLMVHKR
RGDÂLTSHER
RVDÂLEAHRR
REDV̂LIRHGK

(iii) GCGG**C**GGCG

-1123456789
RGPD̂LARHGR
REDV̂LIRHGK

Figure 1B

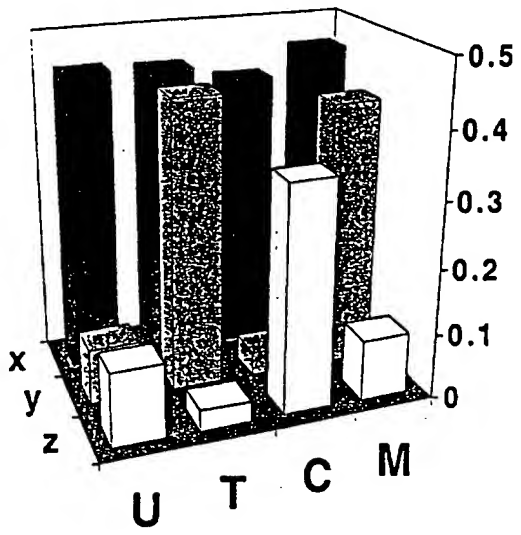


Figure 1c

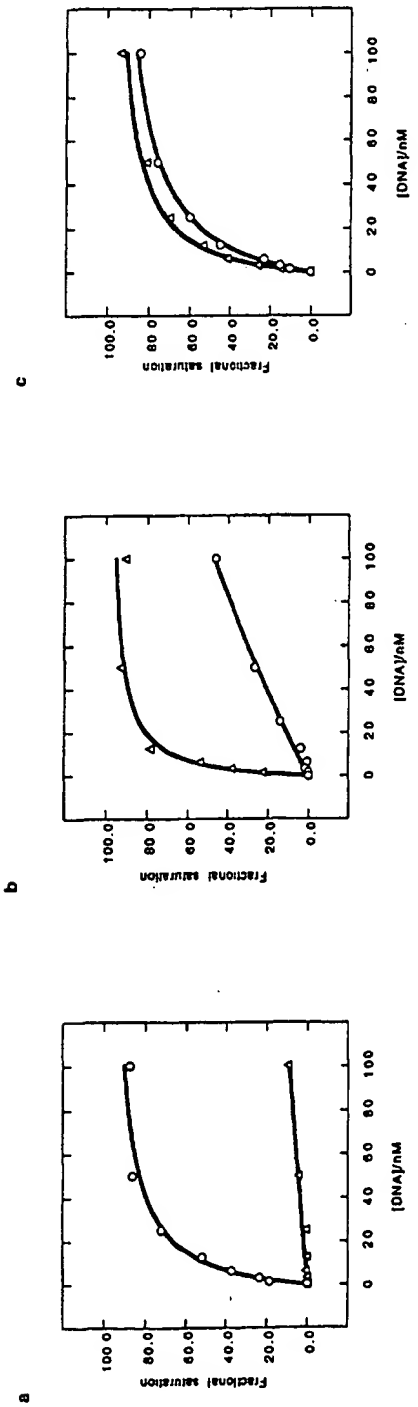

a
b
c
Fractional saturation
0.0
20.0
40.0
60.0
80.0
100.0
0
20
40
60
80
100
[DNA]/nM

Figure 2